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THE FRACTIONATION OF PGB SUB X BY MOLECULAR EXCLUSION CHROMATOG--ETC(U)
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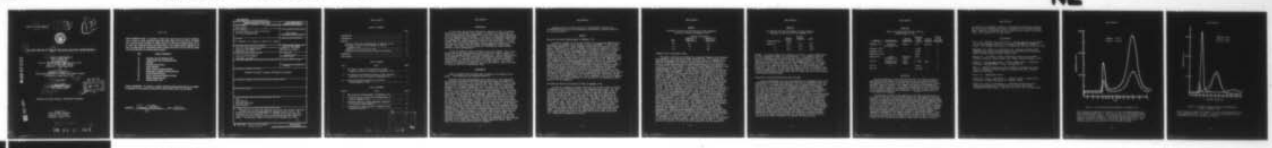

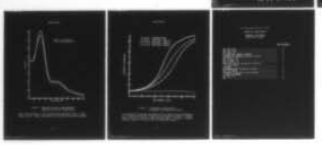

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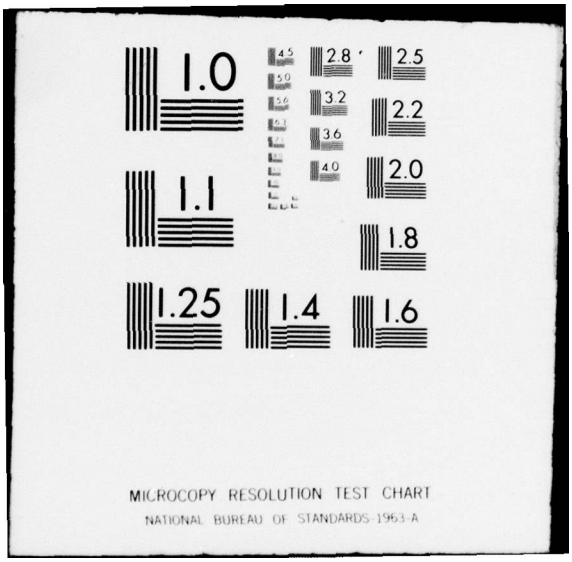
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Preparations of PGB_X were separated into active and inactive fractions by molecular exclusion chromatography on Sephadex G-100 or G-150. The optimum pH for this separation was 7.2. Similar results were found with some other molecular exclusion type packings having equivalent porosity ranges. The molecular weight of the most active fraction was 1539.

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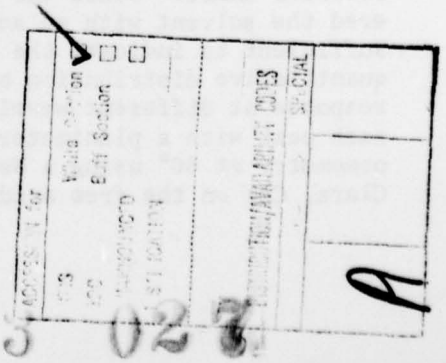

INTRODUCTION

In 1973, Polis et al[1] first reported the synthesis of PGB_X, a polymeric derivative of PGB_1 with the unique property of conserving mitochondrial oxidative phosphorylation under degenerative conditions, that usually resulted in a loss of phosphorylation efficiency. Later Polis and Angelakos[2] demonstrated that PGB_X markedly enhanced the survival of monkeys in cardiogenic shock from an experimentally induced myocardial infarction with fibrillation. In later studies, Polis and Kolata[3] showed that rabbits treated with PGB_X had a 70 percent complete recovery from the effects of experimentally induced global ischemia. More recently these findings were confirmed by similar results obtained in other laboratories[4-6].

As yet the chemical structure of PGB_X has not been defined. This is primarily because PGB_X preparations prepared to date are a complex mixture of polymers of varying molecular sizes and configurations. Such a complex mixture is not amenable to the usual analytical techniques for molecular structure determination. To overcome this difficulty, studies have been undertaken to isolate and purify the active principle in the PGB_X complex. This report describes the separation of PGB_X into an active and inactive fraction by means of molecular exclusion chromatography (MEC).

EXPERIMENTAL

PGB_X was prepared from 15-diketo PGB_1 according to the method of Polis et al[7]. This preparation was designated Batch 26.

Molecular exclusion chromatography was carried out at room temperature with adsorbents equilibrated with the specific mobile phase according to the manufacturer's directions. For analytical MEC, glass columns (1 x 50 cm) were slurry packed with the adsorbent under gravity. Flow rates for compressible packings, e.g., Sephadex, (Pharmacia, Inc., Piscataway, NJ) were maintained between 0.4-0.5 ml per minute by means of a constant pressure Mariotte flask reservoir, while flow rates for the noncompressible packings, e.g., Controlled Pore Glass beads (Corning Glass, Corning, NY), were maintained with a positive-displacement solvent delivery pump (Minipump, Laboratory Data Control, Riviera Beach, FL). To determine the flow rate when compressible packings were used, the effluent was collected in a fraction collector fitted with a 5-ml siphon collector tube and event marker, so that as each 5 ml was collected the event was printed on the strip chart recorder. The flow rate was then calculated from the chart speed and the distance between fraction index marks. The UV absorbance of the effluent stream was monitored on-line, with a UV absorbance detector (Duomonitor, Laboratory Data Control, Riviera Beach, FL) and plotted on the same recorder chart. Since the positive displacement pump used in this study delivered the solvent with an accuracy of 1 percent, then the chart speed alone was sufficient to indicate the effluent flow rate and the eluted volumes. The quantitative distribution of the separated fractions as well as the relative response at different wavelengths were calculated by measuring the area under each peak with a planimeter. Molecular weights were determined by vapor pressure osmometry at 60° using a Wescan Molecular Weight Apparatus (Wescan, Santa Clara, CA) on the free acids dissolved in methanol.

In vitro assays of the PGB_x activity in the separated fractions were carried out with aged rat liver mitochondria preparations by methods described in a previous report[7,8].

RESULTS

Molecular Exclusion Chromatography on Sephadex G-100

In previous attempts to purify PGB_x by MEC, Polis et al used Sephadex LH-20 in methanol[7] and Sephadex G25 in aqueous 25 percent methanol[6]. In both studies PGB_x chromatographed as a single component indicating no fractionation had occurred. Recently in the course of studies on the possible synergistic action of PGB_x and serum albumin on the in vitro PGB_x mitochondrial effect, it was found necessary to separate PGB_x from serum albumin in mixtures of the two. Since Sephadex G-100 is commonly used to separate serum albumin from low molecular weight substances, this technique was used to solve the above separation problem. In addition, since alcohols cannot be used with proteins at room temperature, the above separation was studied in an aqueous medium at neutral pH. When the sodium salt of PGB_x alone was chromatographed on Sephadex G-100 in 0.05 M phosphate buffer pH 7.2, two distinct chromatographic peaks were found (figure 1). The component eluted with the void volume suggesting an apparent molecular weight of between 5000-100,000, based on the elution profile of proteins and dextrans. The retained components therefore exhibited a smaller molecular weight, i.e., <5000. In addition, the distribution of the two components based on their area measurements were 18 percent for the first component and 82 percent for the second. The areas of the two components at λ254 nm and λ280 nm were calculated, and the ratio of area λ254 nm/area λ280 nm was 2.32 for component 1 and 2.60 for component 2, indicating very little difference in the structure of the chromophoric groups found in the two components.

The Effect of pH on Gel Filtration of PGB_x on Sephadex G-100

In order to determine the optimal conditions for the MEC of PGB_x on columns of Sephadex G-100, the effect of pH on this separation was studied. This was carried out by first equilibrating the column with the buffer under study, by passing 10 column-packing volumes of the buffer through the column and then injecting the PGB_x for MEC. In this study the pH range investigated was between pH 6.0 and pH 8.0, because below pH 6.0, PGB_x began to precipitate out and above pH 9.0, PGB_x was slowly inactivated. Therefore, three 0.05 M phosphate buffers: pH 6.0, pH 7.2 and pH 8.0 were used to define the effect of pH on the MEC of PGB_x. These results are shown in figure 2. At pH 8.0 only one component was found, which eluted in the range of the low molecular weight substances. At pH 7.2 and pH 6.0 two components were found, one eluted in the high molecular weight range while the other eluted in the low molecular weight range. In addition, the ratio of the concentration of the high to low molecular weight substances at pH 6.0 was almost the inverse of that found at pH 7.2. These data are summarized in table I.

Table I

The Effect of pH on the Distribution of PGB_x Components From Gel Filtration on Sephadex G-100

pH	Percent Distribution Component #1 (High M.W.)	Component #2 (Low M.W.)
6.0	84	16
7.2	18	82
8.0	0	100

Preparative Gel Filtration of PGB_x

The results obtained on the analytical columns of Sephadex G-100 indicated that component 1 had a molecular weight exceeding 5000. This finding was in complete disagreement with previous gel filtration studies on Sephadex LH-20[7] and G-25[6] in methanol and 25 percent aqueous methanol, as well as from the molecular weight determinations by vapor pressure osmometry using methanol as a solvent[7,8]. In order to decide if the gel filtration separation obtained in the aqueous medium was indeed real, a large amount of PGB_x (200 mg) had to be chromatographed so that enough of each fraction (100 mg) could be isolated for molecular weight measurements. The PGB_x (200 mg) from preparation 26 was dissolved in 1.0 ml of 0.05 M phosphate buffer pH 7.2 and applied to the top of a preparative Sephadex G-100 column (2.5 x 50 cm). The solution was allowed to enter the column by gravity. The sides of the column were then rinsed with buffer, and the washings also allowed to enter the column. The washing step was carried out two more times and finally the buffer was layered over the top of the column and the buffer reservoir attached. During the sample addition and washing steps, extreme care was taken so that no air entered the column packing. The column effluent was monitored by recording the UV absorbancy at λ254 nm. From this recording, fractions of the effluent stream were combined in order to obtain PGB_x separated fractions 1 and 2 in purified form. Because of the overlap of the chromatographic peaks in the column eluate during gel filtration, three combinations were made: combination (a) contained component 1, combination (b) contained a mixture of components 1 and 2, and combination (c) contained component 3. In order to desalt and/or concentrate the purified PGB_x components for further study, each combination was acidified with HCl to pH 3.0 and extracted into isobutanol. The isobutanol layer was separated and washed with water; 3 water extractions were sufficient to remove the contaminating carrier buffers and excess acid. Finally the isobutanol layer was separated and evaporated at 50° under reduced pressure. The dried PGB_x fractions were weighed and then dissolved in methanol for molecular weight measurements. Following these measurements the solutions were recovered, the methanol evaporated off and the residues dissolved in ethanol for UV absorption spectral analysis. For biological assay in aged mitochondria, aliquots of the PGB_x fractions in ethanol were diluted in $NaHCO_3$ at final concentration of 2.5 mM $NaHCO_3$ and 25 percent ethanol. The data for the weight of PGB_x recovered in each fraction and their molecular weights are shown in table II. Figure 3 shows the UV spectra of the PGB_x fractions separated by MEC and measured in

Table II

The Recovery and Molecular Weights of PGB_x Fractions Separated by Gel Filtration on Sephadex G-100

	Weight	Percent Recovery	M.W.
Preparation #26	200 mg		
Fraction 1	58.8	29.5	2437
2	91.7	46.0	2384
3	48.9	24.5	1539

ethanol. The spectra appear to be identical in that on a similar weight basis all the UV absorption curves are super-imposable. It is apparent from this that the chromophoric groups responsible for the UV absorption of PGB_x are similar if not identical to those in the MEC separated fractions. Figure 4 shows the results of PGB_x assays in which the phosphorylation ability of aged mitochondria was restored by additions of PGB_x. The figure covers the range of 1-5 μg of test sample and includes the results of the standard PGB_x for comparison. Fraction 1, the high molecular weight fraction is completely inert in this in vitro system. On the other hand fractions 2 and 3 are active, with fraction 3, the lowest molecular weight fraction being more active than fraction 2 with median molecular weight.

Evaluation of Various Molecular Exclusion Packings

A major disadvantage in the use of ME packings of the Sephadex type, is the compressibility of the gel that eventually slows down or completely blocks the solvent flow. To overcome this difficulty only low pressure heads may be used with the result that gel filtration chromatography becomes time consuming. Even using low pressure heads the gel gradually compresses so that constant flow rates cannot be maintained. Therefore for quantitative area measurements the column flow must be continually monitored by, for example, the system described under "Experimental." A survey of various molecular exclusion packings was then undertaken looking for a packing material that would give satisfactory chromatographic resolution of PGB_x components, yet would not be compressible under the pressure of more rapid flow rates. The results of this survey are shown in table III that lists the packing, the manufacturer, the composition, the manufacturer's exclusion limits, the system used for solvent delivery and the number of components found on MEC. These results show that in the Sephadex series only the G-100 and G-150 in the molecular exclusion range of 4-150K and 5-300K were effective in separating PGB_x fractions. Sephadex G packings with smaller and larger exclusion limits were not effective. On the other hand, separations of PGB_x were also achieved with Controlled Pore Glass packings CPG 125 and 175 having exclusion limits of 0.5-35K and 11-60K while CPG 240 with exclusion limits 2.5-125K did not separate the PGB_x fractions.

Table III

Survey of Packings Suitable for Exclusion Chromatography of PGB_x

Packing	Manufacturer	Packing Composition	Molecular Weight Range	Solvent Delivery	Number of PGB_x Components
Sephadex G-25	Pharmacia (Piscataway, NJ)	Dextran	1-5K	gravity	1
Sephadex G-100	"	"	4-150K	"	2
Sephadex G-150	"	"	5-300K	"	2
Sephracryl G-200	"		3-60K	"	1
Biogel P-60	Biorad (Richmond, CA)	Polyacryl-amide	3-60K	"	1
CPG 125	Corning Glass (Corning, NY)	Porous glass	0.5-35K	pump	2
CPG 175	"	"	11-60K	"	2
CPG 240	"	"	2.5-125K	"	1

DISCUSSION

The data presented in this study show that MEC is an effective means of separating the PGB_x complex into biologically active and inactive fractions. The importance of this lies in the fact that the active fraction has a smaller molecular weight and should be less complex and therefore more amenable to molecular structure analysis. The possible incorporation of this methodology into the general scheme for the preparation of PGB_x must now await the assay, of the *in vitro* biologically active material, in *in vivo* systems similar to those used by Polis *et al*[2,3].

The mechanism by which the ME separation of the PGB_x fractions occurs is not clear at this time. From the results shown here the "apparent" molecular weight of the PGB_x fraction measured by MEC must be different in aqueous medium from that in methanol, since the PGB_x fraction emerging from the Sephadex column in the void volume should have a molecular weight in the gel exclusion limits (4-300K), while this fraction when analyzed by vapor phase osmometry in methanol gives a value of 2437. It may be hypothesized that in aqueous media, hydrogen bonding of the small polymers may result in the formation of a complex exhibiting a high molecular weight and, that in methanol the hydrogen bonding is suppressed; consequently, the complex exhibits a low molecular weight. Alternatively, the failure of the "larger molecule" of PGB_x to enter the pores of the gel may be a function of the shape of the molecule in that it is stretched linearly so that the molecule physically cannot enter the pores of the gel.

In alcohol this stretching is reduced, or the molecule becomes more globular, and thus can pass through the gel pores to emerge in the low molecular weight range. Whatever the mechanism involved, aqueous MEC does afford an additional step in the elucidation of the PGB_x complex.

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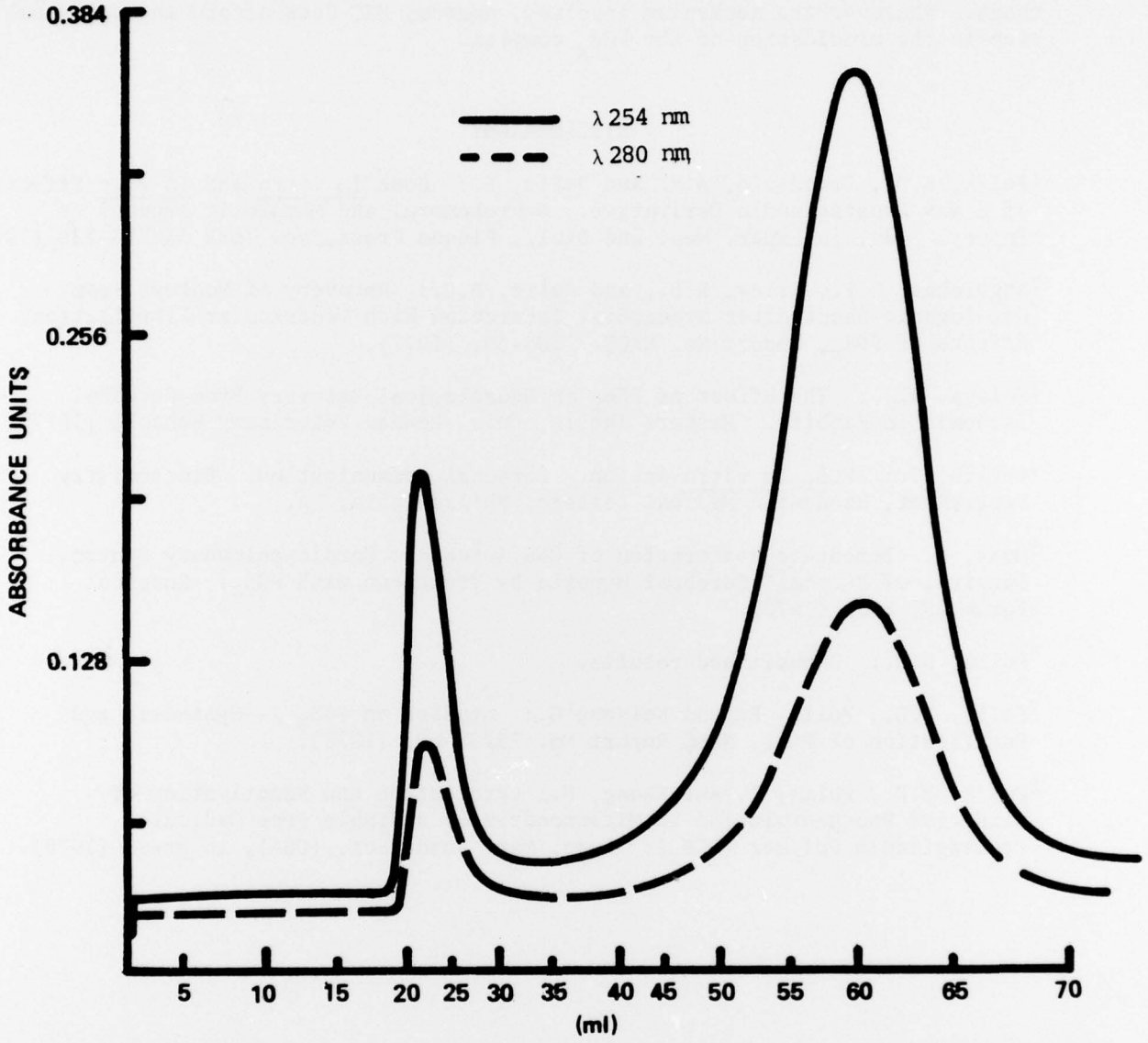

Figure 1 - PGB_x Exclusion Chromatography of Sephadex G-100.

PGB_x (0.66µg) was injected on a column (1 x 50 cm) and chromatographed with 0.05 M phosphate buffer pH 7.2. The flow rate was 0.5 ml per minute measured with a 5 ml volumetric siphon. The collected fractions are indicated on the record by the volume of effluent. The effluent was monitored with a 2 wave length UV detector at λ254 nm (solid line) and λ280 nm (broken line). The detector was set at 0.64 AUFS on a 10 mv strip chart recorder.

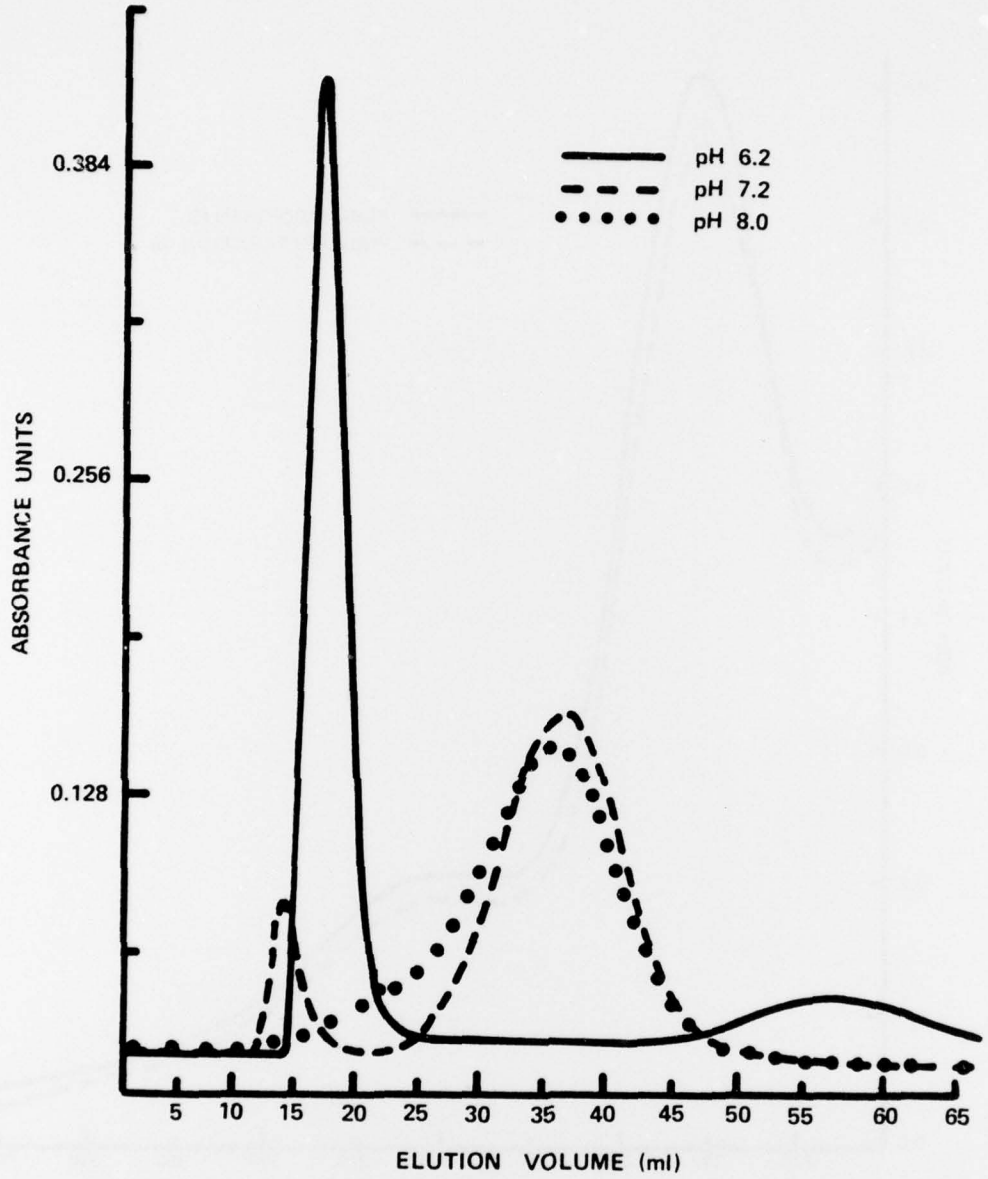

Figure 2 - The Effect of pH on Exclusion Chromatography of PGB_x on Sephadex G-100.

PGB_x (0.66μg) was injected on column (1 x 50 cm) and chromatographed with 0.05 M phosphate buffer: (A) pH 6.0, (B) pH 7.2, and (C) pH 8.0. The experimental details are the same as in figure 1.

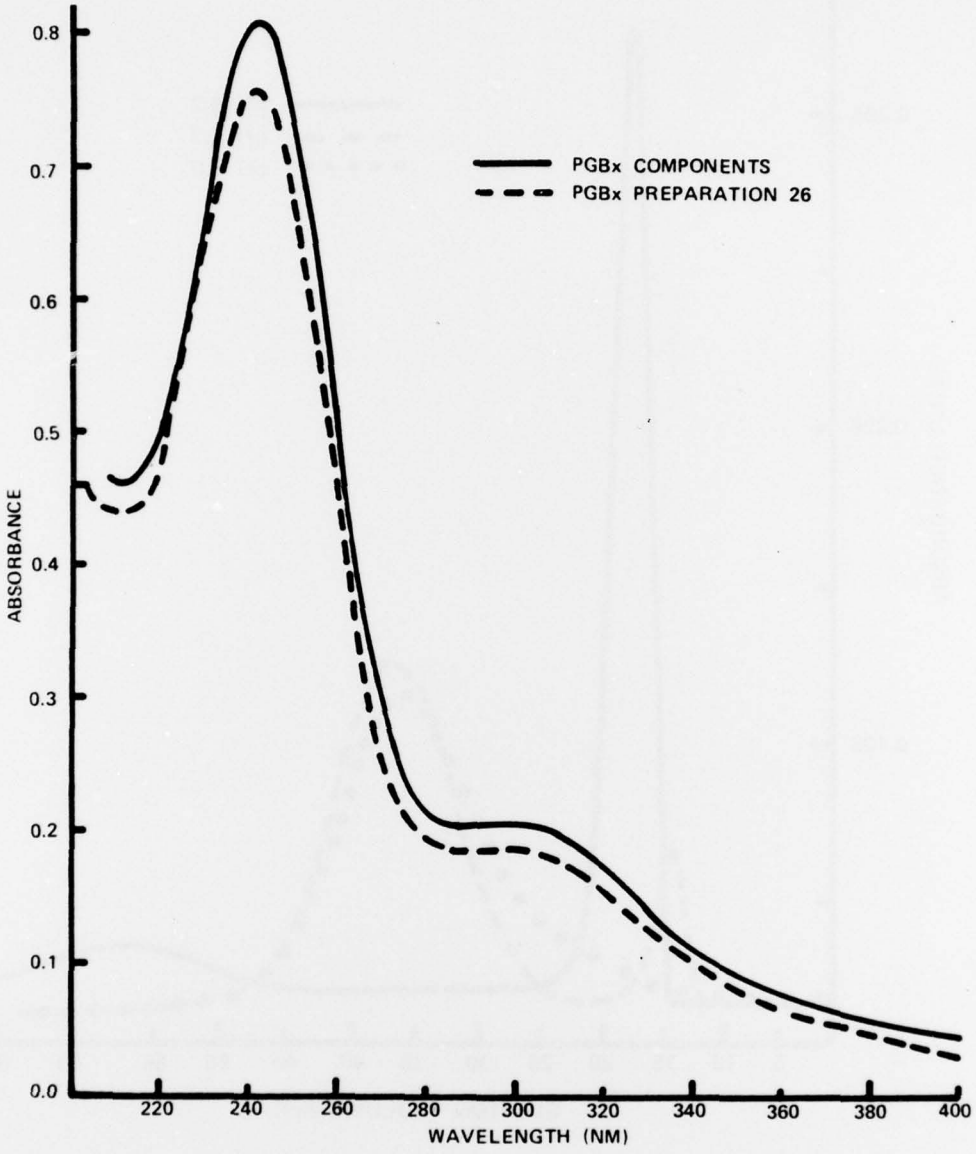

Figure 3 - Absorption Spectra of PGB_X Components Separated by Exclusion Chromatography

Spectra were measured in a Cary recording spectrophotometer using 1 cm path length cuvettes. Sample concentration was 30 µg per ml dissolved in ethanol.

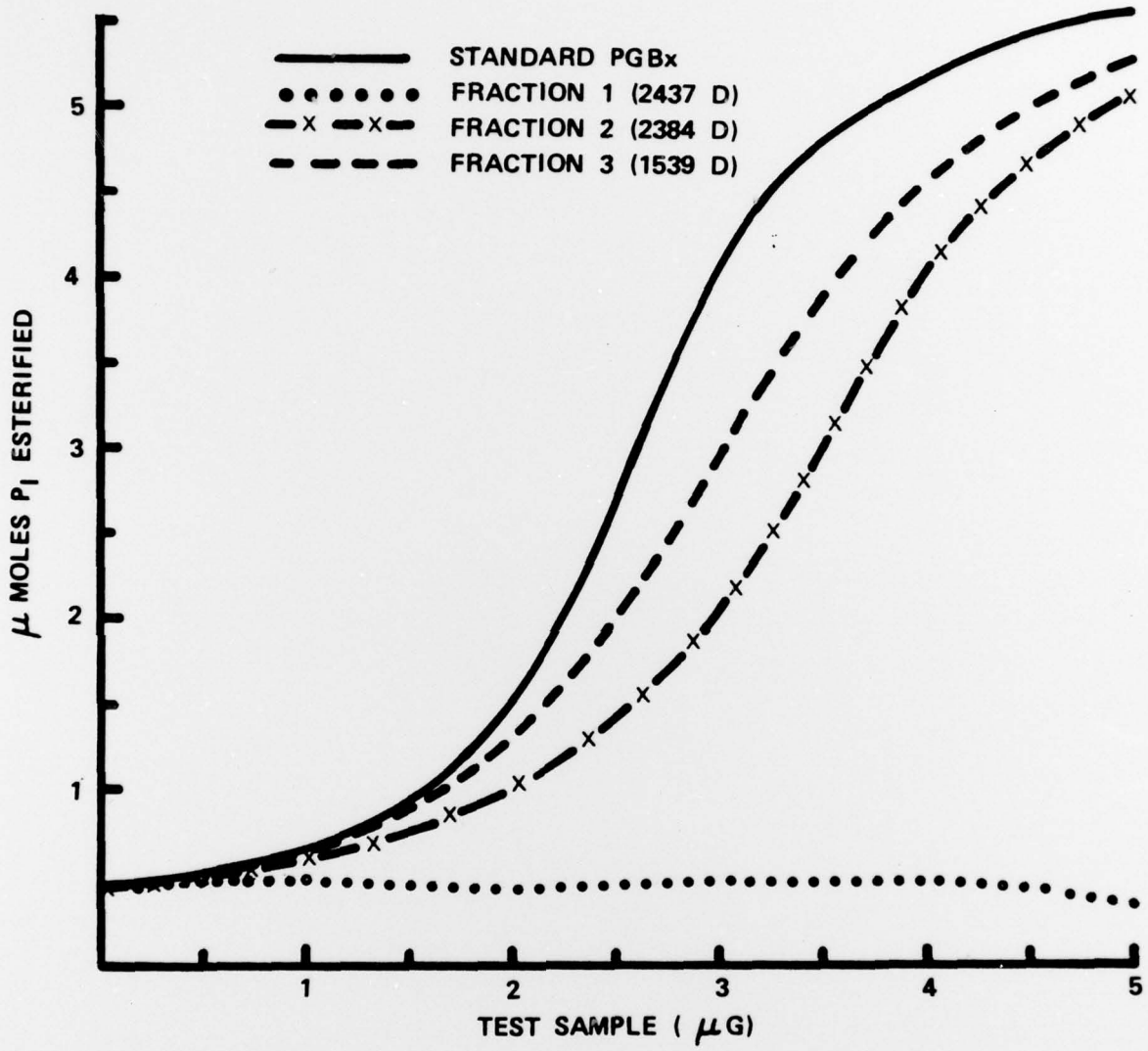

Figure 4 - The Recovery of PGB_X Activity in Sephadex G-100 Separated Fraction.

Test conditions for recovery of phosphorylation of aged rat liver mitochondria by the addition of PGB_X were carried out as previously described[7,8]. Standard PGB_X is solid line; Fraction 1 (high molecular weight)...; Fraction 2 (medium molecular weight)-x-x; and Fraction 3 (low molecular weight)---.

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